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Activation of the canonical Wnt/β-catenin pathway enhances monocyte adhesion to endothelial cells

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Abstract

Monocyte adhesion to vascular endothelium has been reported to be one of the early processes in the development of atherosclerosis. In an attempt to develop strategies to prevent or delay atherosclerosis progression, we analyzed effects of the Wnt/β-catenin signaling pathway on monocyte adhesion to various human endothelial cells. Adhesion of fluorescein-labeled monocytes to various human endothelial cells was analyzed under a fluorescent microscope. Unlike sodium chloride, lithium chloride enhanced monocyte adhesion to endothelial cells in a dose-dependent manner. We further demonstrated that inhibitors for glycogen synthase kinase (GSK)-3β or proteosome enhanced monocyte-endothelial cell adhesion. Results of semi-quantitative reverse transcriptase polymerase chain reaction(RT-PCR) indicated that activation of Wnt/β-catenin pathway did not change expression levels of mRNA for adhesion molecules. In conclusion, the canonical Wnt/β-catenin pathway enhanced monocyte-endothelial cell adhesion without changing expression levels of adhesion molecules.

Keywords: Atherosclerosis; β-Catenin; Endothelial cells; Monocyte; Wnt

Atherosclerosis, the primary cause of heart disease and stroke, is the main cause of death in western societies [1,2]. Monocytes adhere to endothelium in the pathophysiolocal condition and transmigrate into the subendothelial region where they become foam cells [3,4]. Lipid-laden foam cells do not undergo the complete apoptotic process due to decreased phagocytosis by macrophages in the atherosclerotic lesion [5], resulting in secondary necrosis of foam cells and release of pro-inflammatory debris [6–8]. A chronic inflammatory condition of atherosclerosis can lead to a fatal clinical event by plaque rupture and thrombosis.

Adhesion of monocytes to artery walls is a hallmark of atherosclerosis. Accumulation of minimally oxidized low-density lipoprotein (LDL) up-regulates low-density lipoprotein receptor-1 (LOX-1) and stimulates endothelial cells to produce pro-inflammatory molecules, including adhesion molecules, chemotactic molecules, and growth factors [9,10]. Leukocyte rolling along the endothelial surface is the first step of monocyte adhesion. This step is mediated by P- and E-selectins that bind to carbohydrate ligands on leukocytes [3,11]. The firm adhesion of monocytes to endothelium is mediated by the integrins on monocytes that interact with cell adhesion molecules on the endothelium. The integrins αLβ2 (lymphocyte function-associated [LFA]-1) and αMβ2 (Mac-1) on monocytes bind to the intracellular adhesion molecule (ICAM) on the endothelial surface [12]. The integrin α4β1 (very late activation antigen [VLA]-4) on mono-

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cytes interacts with vascular cell adhesion molecule-1 (VCAM-1) on the endothelium [13].

Several signaling pathways have been reported to regulate monocyte adhesion to endothelial cells [8,14,15]. Activation of RhoA GTPase and nuclear factor (NF)-κB pathway has been demonstrated to up-regulate adhesion molecules and enhance monocyte adhesion to endothelial cells. A recent study demonstrated that statins down-regulate RhoA GTPase and reduce monocyte adhesion to endothelial cells [14]. However, there have been no studies that report effects of the Wnt signaling pathway on monocyte adhesion to endothelial cells, even though β-catenin has been known to play an important role in both cell–cell adhesion and in the Wnt signaling pathway. The purpose of this study was to evaluate and analyze mechanisms by which the Wnt/β-catenin signaling pathway regulates monocyte adhesion to endothelial cells.

The Wnt signaling pathway plays a key role in numerous developmental processes [16]. Wnts are secreted glycoproteins that bind to transmembrane frizzled receptors. Various secreted factors, such as cerberus and dickkopf, block interaction of Wnt with the receptors/co-receptors [17]. The Wnt pathway diversifies into three branches: the β-catenin/GSK-3β pathway, the Wnt/Ca^{2+} pathway, and the planar cell polarity pathway [18].

A key effector of the Wnt signaling pathway is β-catenin, a protein initially discovered for its role in cell adhesion [19]. In the absence of Wnts, β-catenin is associated with a cytosolic multi-protein complex consisting of adenomatous polyposis coli, GSK-3β, and axin [18]. Interaction of Wnts to the receptor disrupts the cytosolic multi-protein complex and inactivates casein kinases Iα/Iε and GSK-3β. GSK-3β phosphorylates β-catenin at serine and threonine residues in the NH_2-terminal region, resulting in ubiquitination and proteosome-mediated degradation of β-catenin [20]. Stabilized β-catenin translocates into the nucleus and associates with T-cell factor (TCF), which leads to transcription of the Wnt target genes [21]. In the absence of β-catenin, TCF interacts with co-repressors and represses transcription.

In addition to its role in the signaling pathway, β-catenin is a component of cell–cell adhesion junction that promotes cell adhesion [19]. The β-catenin binds to the intracellular domain of transmembrane protein cadherin, a Ca^{2+}-dependent homotypic adhesion molecule, and links cadherins to the actin cytoskeleton through an adaptor protein α-catenin to promote cell adhesion and control cell shape [22,23]. The adhesion function of β-catenin depends on a subcellular pool of β-catenin that is membrane-associated and stable.

The β-catenin is a dual-function protein that coordinates cell adhesion and the cell signaling pathway. Inappropriate cell signaling of the β-catenin pathway has been linked to loss of cadherin-mediated adhesion during tumor cell invasion and metastasis [24,25]. A recent study demonstrated that dismantling of cadherin-mediated cell–cell contacts stimulated proliferation of smooth muscle cells in the saphenous vein and released β-catenin for intracellular signaling pathway [26].

Materials and methods

Human endothelial cells. Human umbilical vein endothelial cells (HUVEC) and human coronary arterial endothelial cells (HCAEC) obtained from healthy adults were purchased from Cambrex Bioscience (Walkersville, MD). Human endothelial cells were maintained in endothelial growth medium-2MV (EGM-2MV) according to the manufacturer's instructions (Cambrex Biosciences). EGM-2MV is composed of basic endothelial medium-2 and supplements (human epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor-b, insulin-like growth factor-1, gentamycin/amphotericin-B, hydrocortisone, 5% fetal bovine serum (FBS), and ascorbic acid). Human endothelial cells were grown in a humidified incubator with 5% CO_2 at 37 °C. All human endothelial cells used in this study were within 3rd to 7th passages.

Labeling of human monocyte THP-1 with fluorescent dye. Human monocyte THP-1 cells were purchased from ATCC and maintained at $1–8 \times 10^5$ cells/ml in Advanced RPMI1640 (Invitrogen) supplemented with 7% FBS, 10 mmol/l Hepes/pH 7.5, 2 mmol/l Glutamax, and 100 U/ml penicillin/streptomycin. THP-1 cells, grown in suspension, were centrifuged, rinsed with phosphate-buffered saline (PBS) twice, and labeled with a fluorescein dye Calcein AM (Molecular Probes), according to the manufacturer's instruction. Fluorescein labeled THP-1 cells were centrifuged, rinsed with PBS twice, resuspended in EGM-2MV at 5×10^6 cells/ml, and used immediately for cell adhesion assay.

Cell adhesion assay. When human endothelial cells grown in 48-well plates reached near 100% confluency, 190 μl of fluorescein labeled THP-1 cells (5×10^6 cells/ml) was added to each well and further incubated for 20–90 min in a humidified incubator with 5% CO_2 at 37 °C. Various concentrations of lithium chloride (LiCl), sodium chloride (NaCl), MG132 (Sigma), highly specific GSK-3 inhibitor (Calbiochem), or protein kinase C (PKC) inhibitor (Calbiochem) were added according to the experimental conditions. Dimethyl sulfoxide (DMSO) was used to dissolve kinase inhibitors or MG132. Equal amounts of DMSO were added where no inhibitors were added. THP-1 cells were removed and endothelial cells were gently rinsed three times with PBS. THP-1 cells attached to endothelial cells were analyzed under the fluorescence microscope (final magnification 40×). All images from the fluorescence microscopic analyses were taken using CoolSNAP ES camera with 155 ms exposure for consistency. At least three independent experiments were performed for each cell adhesion assay.

Semi-quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR). Total RNAs from THP-1 cells (6×10^6 cells) or human endothelial cells grown on 60 mm culture dishes incubated in media containing GSK-3β inhibitor (final concentration 8 μmol/l) or DMSO for 1 h were isolated using Absolutely RNA Miniprep kits (Stratagene), according to the manufacturer's instructions. Complementary DNAs (cDNAs) from total RNA samples (200 ng each) were synthesized in 25 μl at 60 °C using gene specific primers and ThermoScript RT-PCR system (Invitrogen), according to the manufacturer's instructions. PCR products (30 cycles) obtained from 2 μl of each cDNA sample was separated on 1.8% SeaPlaque agarose gels (Cambrex) and stained by SYBR Green I. Expression levels of adhesion molecules for integrin β1, integrin β2, VCAM-1, ICAM-1, and β-actin were determined using densitometry. The expression level of β-actin was used as an internal control of messenger RNA (mRNA) concentrations in each sample.

Statistical analysis. Means and standard errors of the means (SEM) were calculated. Statistical comparison of data was determined using one-way or two-way analysis of variance (ANOVA), followed by the Bonferroni post-test adjustment. A value of $P < 0.05$ was considered significant. Data are presented as means ± SEM. Numbers of experiments performed for each cell type and assay are presented as n.

Results

Lithium, an activator for the Wnt signaling pathway, enhances monocyte adhesion to various human endothelial cells

Studies using experimental animal models demonstrated that adhesion of circulating monocytes to endothelial cell monolayer is the earliest event in the development of atherosclerosis [27]. Studies so far indicate that adhesion of monocytes to endothelial cells results from both quantitative changes of expression levels and quantitative changes in avidity of adhesion molecules [11]. Various signaling pathways, such as RhoA GTPase and Toll-like receptor/NF-κB pathways, have been reported to regulate monocyte adhesion to endothelial cells [8,14,15]. In this study, we report the effects of GSK-3β/β-catenin signaling pathway on monocyte adhesion to various human endothelial cells.

Lithium (Li^+), a therapeutic agent used to treat bipolar disorder, has been shown to regulate gene expression and embryonic development/patterning by activating the Wnt signaling pathway [28,29]. In order to determine effects of the Wnt signaling pathway on development of atherosclerosis, various concentrations of LiCl or NaCl were added to co-incubation of endothelial cells and monocytes. As shown in Fig. 1, LiCl enhanced adhesion of monocytes to HUVEC and HCAEC in a dose-dependent manner. NaCl, as a control, did not enhance monocyte adhesion to endothelial cells. The effect of LiCl at concentrations higher than 0.2 mmol/l on monocyte adhesion to endothelial cells was statistically significant ($P < 0.02$, $n = 3$). The difference of the LiCl effect between vein and artery endothelial cell types was statistically insignificant ($P > 0.05$, $n = 3$). We chose HCAEC for this study, because development of atherosclerosis in coronary arteries leads to coronary artery disease. The results in Fig. 1 demonstrate that incubation of endothelial cells and monocytes in the presence of LiCl for 60 min enhanced monocyte adhesion to endothelial cells. This is a novel study revealing a possible role of the Wnt signaling pathway in monocyte adhesion to endothelial cells.

The β-catenin pathway, a canonical Wnt signaling pathway, regulates monocyte adhesion to endothelial cells

The Wnt signaling pathway diversifies into a canonical Wnt signaling pathway (GSK-3/β-catenin) and non-canonical Wnt pathways (Ca^{2+} pathway and RhoA GTPase/cytoskeleton pathway). In order to identify a branch of the signaling pathway that regulates monocyte adhesion to endothelial cells, we used an inhibitor highly specific for GSK-3β or PKC (Calbiochem). When monocytes and endothelial cells were co-incubated in the medium containing various concentrations of GSK-3β inhibitor for 60 min, the GSK-3β inhibitor enhanced monocyte adhesion to endothelial cells in a dose-dependent manner (Fig. 2A and B). The effect of GSK-3β inhibitor was statistically significant at concentration 0.3 μmol/l ($P < 0.05$, $n = 4$). However, co-incubation of monocytes with endothelial cells in the presence of PKC inhibitor showed no statistically significant effect on monocyte adhesion to endothelial cells at all the concentrations tested (Fig. 2C). This result indicates that the canonical Wnt/β-catenin pathway enhances monocyte adhesion to endothelial cells.

Without activation of the Wnt signaling pathway, a cytosolic pool of β-catenin is phosphorylated by GSK-3β and degraded by ubiquitin-mediated proteosome. Activation of the Wnt signaling pathway inactivates GSK-3β and leads to accumulation of β-catenin [19,20]. Thus, we also analyzed the effect of an inhibitor for proteosome (MG132) on monocyte adhesion to endothelial cells. Addition of MG132 enhanced monocyte adhesion

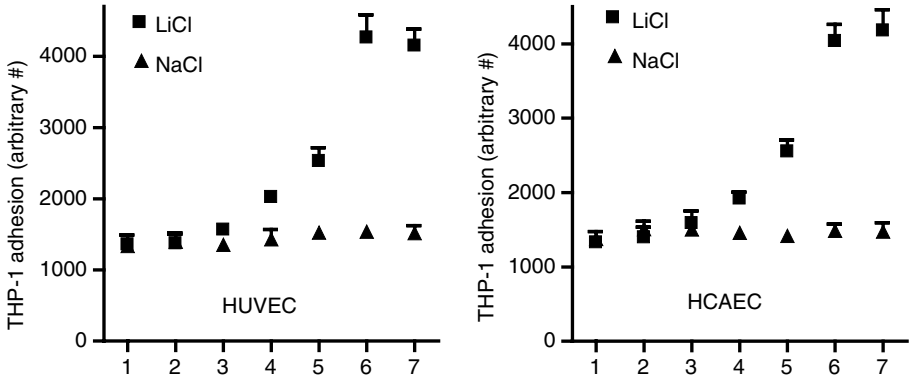

Fig. 1. Lithium, an activator for the Wnt signaling pathway, enhances monocyte adhesion to various human endothelial cells. Human endothelial cells in 48-well plates were incubated with fluorescein-labeled THP-1 in the medium containing various concentrations of LiCl or NaCl for 1 h. Endothelial cells were rinsed with PBS three times. THP-1 cells attached to endothelial cells were analyzed under the fluorescence microscope. Concentrations of LiCl or NaCl used were 0 (lane 1), 0.007 (lane 2), 0.02 (lane 3), 0.07 (lane 4), 0.2 (lane 5), 0.7 (lane 6), and 2 (lane 7) mmol/l. Results are presented as means ± SEM; n (numbers of experiments performed) = 3. $^*P < 0.01$ *vs.* control (lane 1: without LiCl or NaCl). Statistical comparison of data was determined using two-way ANOVA, followed by the Bonferroni post-test adjustment.

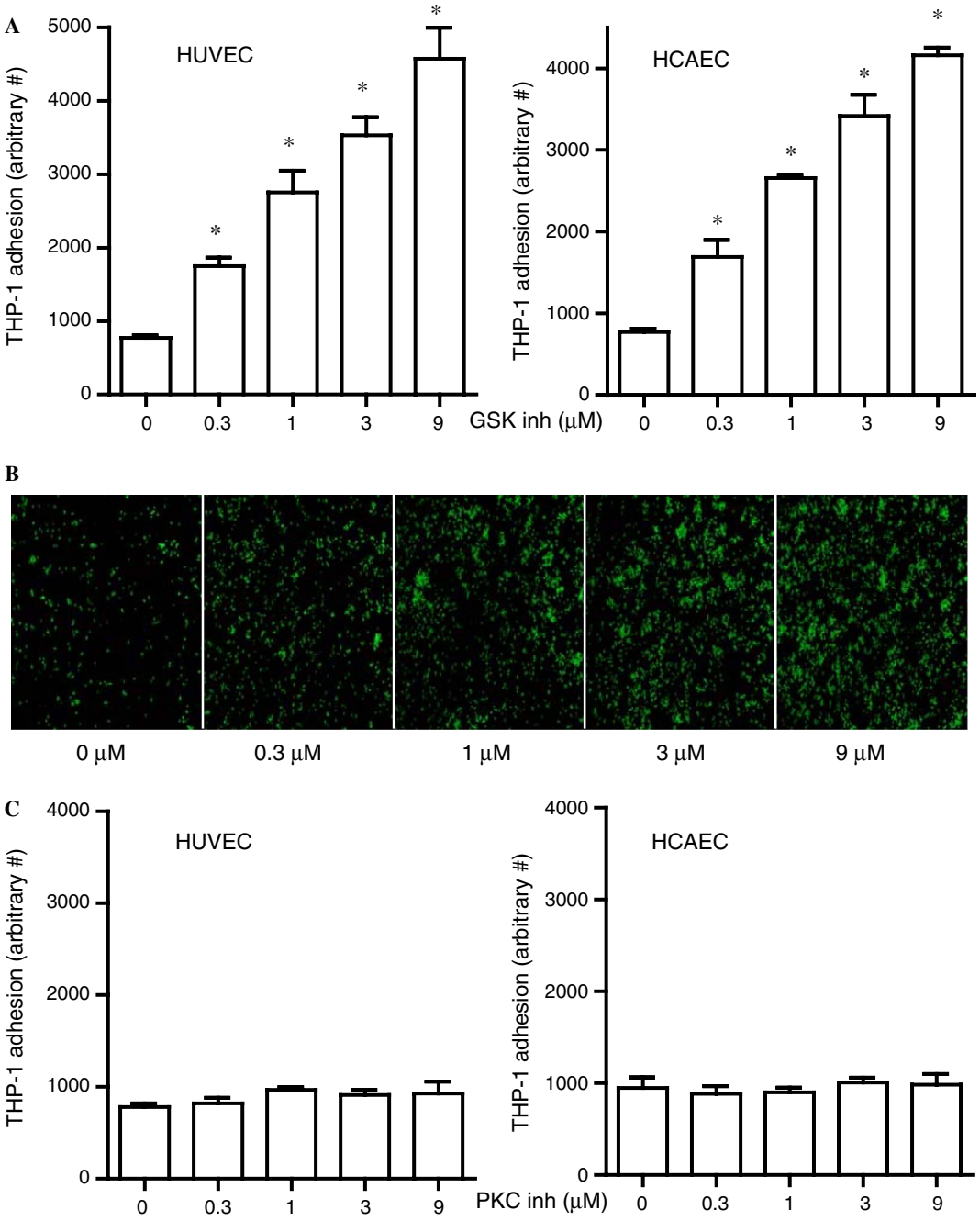

Fig. 2. GSK-3β inhibitor enhances monocyte adhesion to endothelial cells. (A) Effect of GSK-3β inhibitor on monocyte adhesion to endothelial cells. Monocyte-endothelial cell adhesion assay was performed in the medium containing various concentrations of GSK-3β inhibitor. Results are presented as means ± SEM; n (numbers of experiments performed) = 4. *$P < 0.01$ *vs.* control (without GSK-3β inhibitor). Statistical comparison of data was determined using two-way ANOVA, followed by the Bonferroni post-test adjustment. (B) Representative images of THP-1 adhesion to HCAC in various concentrations of GSK-3β inhibitor. Monocytes attached to endothelial cells were analyzed under a Nikon fluorescence microscope (final magnification 40×). All images were taken using CoolSNAP ES camera with 155 ms exposure for consistency. (C) Effect of PKC inhibitor on monocyte adhesion to endothelial cells.

to endothelial cells in a dose-dependent manner (Fig. 3). The effect of MG132 was statistically significant at concentration 0.3 μmol/l ($P < 0.05$, $n = 4$). The similar effects of MG132 and GSK-3β inhibitor shown in Figs. 2 and 3 indicate that activation of the canonical Wnt/β-catenin signaling pathway enhances monocyte adhesion to endothelial cells.

Enhanced monocyte adhesion to endothelial cells by the Wnt/β-catenin pathway does not require synthesis of new mRNAs or proteins

We incubated cells in the medium containing GSK-3β inhibitor for different time-courses to determine the optimal incubation time for monocyte adhesion to endothelial

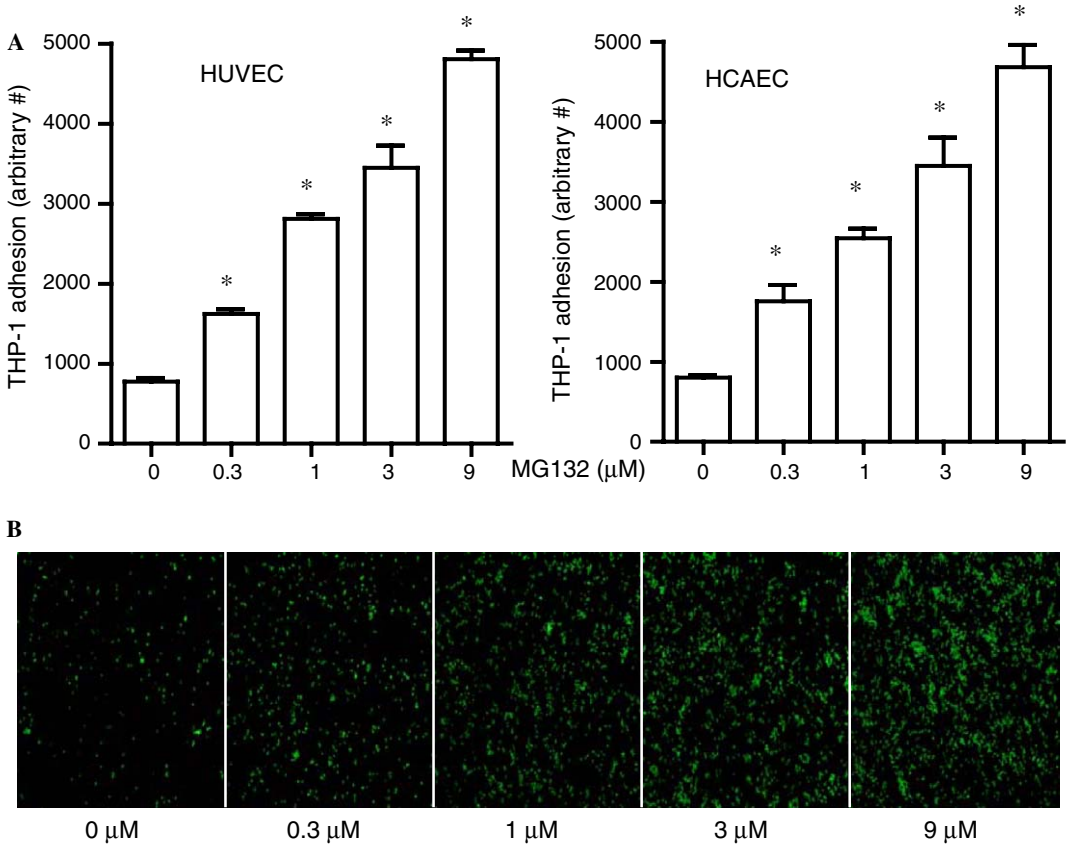

Fig. 3. Proteosome inhibitor MG132 enhances monocyte adhesion to endothelial cells. (A) Effect of MG132 on monocyte adhesion to endothelial cells. Cell adhesion assay of fluorescein labeled THP-1 and endothelial cells in 48-well plates in the medium containing various concentrations of MG132 was performed as described in Materials and methods. Results are presented as means ± SEM; n (numbers of experiments performed) = 4. $^{*}P < 0.01$ *vs.* control (without MG132). Statistical comparison of data was determined using two-way ANOVA, followed by the Bonferroni post-test adjustment. (B) Representative images of THP-1 adhesion to HCAC in various concentrations of MG132. Monocytes attached to endothelial cells were analyzed under a Nikon fluorescence microscope (final magnification 40×). All images were taken using CoolSNAP ES camera with 155 ms exposure for consistency.

cells. As shown in Fig. 4, incubation of cells in the medium containing GSK-3β inhibitor for only 20 min markedly enhanced monocyte adhesion to endothelial cells ($P < 0.001$, $n = 3$). Differences of the GSK-3β inhibitor effect on monocyte adhesion to endothelial cells for incubation times from 20 to 60 min were statistically insignificant (lane 2 *vs.* lane 4 or 6: $P > 0.05$, $n = 3$). When cells were incubated in the medium containing GSK-3β inhibitor for 120 min, the efficacy of GSK-3β inhibitor was lower than those for 20–60 min (lanes 2, 4, and 6 *vs.* lane 8: $P < 0.05$).

Since several pathways have been reported to regulate expression levels of certain adhesion molecules involved in monocyte-endothelial cell adhesion [8,14,15], we further analyzed whether synthesis of new mRNA or proteins is required for the enhanced monocyte adhesion to endothelial cells by the Wnt/β-catenin pathway. Addition of actinomycin D or cycloheximide to the medium did not show statistically significant changes in the effects of the GSK-3β inhibitor on monocyte adhesion to endothelial cells (Fig. 5A). Furthermore, results of semi-quantitative RT-PCR showed that treatment of endothelial cells or

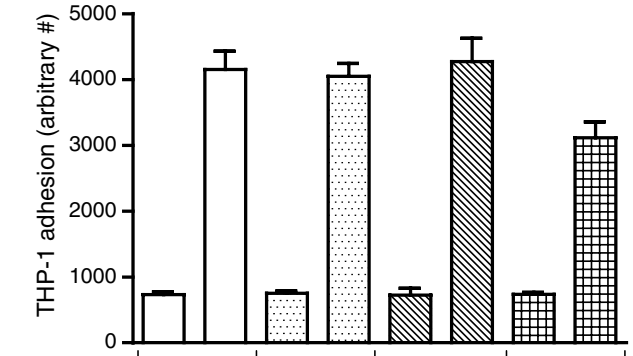

Fig. 4. Effect of incubation time on monocyte adhesion to endothelial cells. Fluorescein labeled THP-1 cells were added to endothelial cells in the presence of GSK-3β inhibitor (final concentration 8 μmol/l) or DMSO, incubated for 20–120 min, and rinsed three times. THP-1 cells attached to endothelial cells were analyzed as described in Materials and methods. Results are presented as means ± SEM; n (numbers of experiments performed) = 3. Statistical comparison of data was determined using one-way ANOVA, followed by the Bonferroni post-test adjustment.

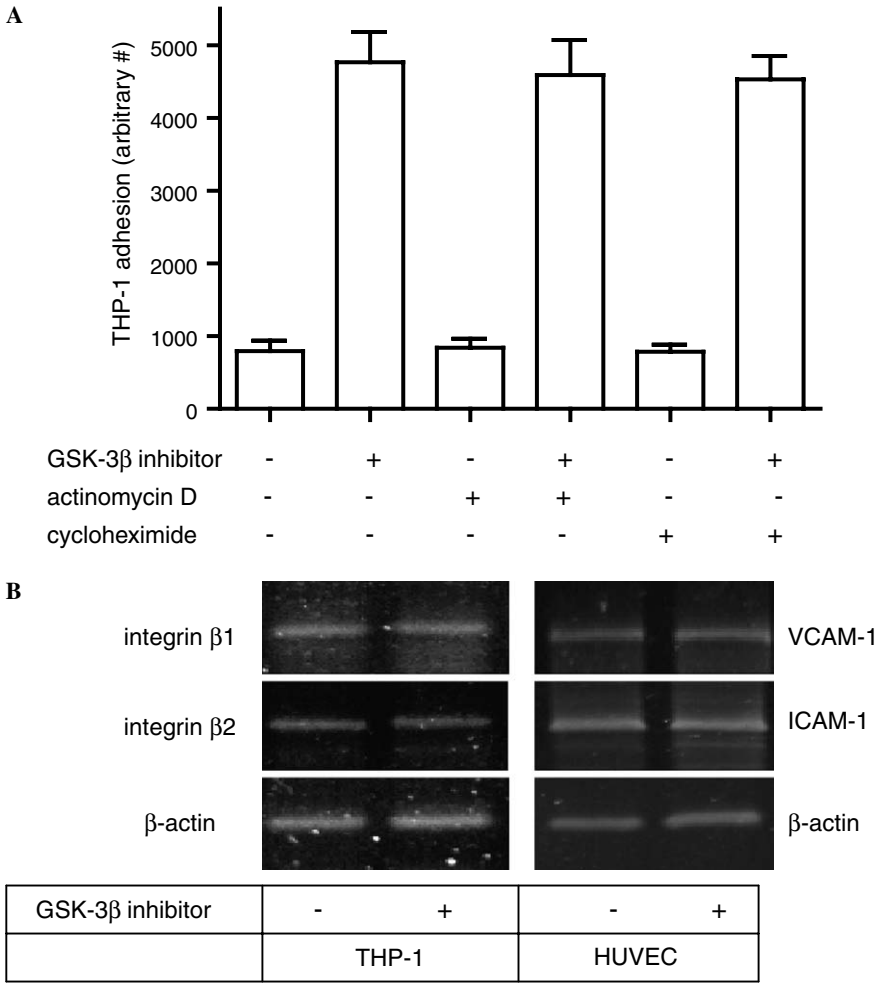

Fig. 5. (A) Effects of actinomycin D or cycloheximid on monocyte adhesion to endothelial cells. Actinomycin D (final concentration 2 μg/ml) or cycloheximide (final concentration 20 μg/ml) was added to fluorescein labeled THP-1 cells just before added to endothelial cells. GSK-3β inhibitor (final concentration 8 μmol/l) was added in THP-1 cell suspension for lanes 2, 4, and 6. THP-1 cells attached to endothelial cells were analyzed as described in Materials and methods. Results are presented as means ± SEM; *n* (numbers of experiments performed) = 3. Statistical comparison of data was determined using one-way ANOVA, followed by the Bonferroni post-test adjustment. (B) Total RNAs from HUVECs and THP-1 were obtained and used for semi-quantitative RT-PCR. The expression level of β-actin was used as an internal control of messenger RNA (mRNA) concentrations in each sample.

monocytes with GSK-3β inhibitor for 1 h did not significantly change expression levels of mRNA for adhesion molecules (Fig. 5B). Results in Fig. 5 suggest that synthesis of new mRNA or protein may not be required for β-catenin-mediated enhanced monocyte adhesion to endothelial cells.

Discussion

Monocyte adhesion to vascular endothelium has been reported to be the earliest event in the development of atherosclerosis. Understanding the basic mechanisms of monocyte adhesion to endothelium may provide opportunities to develop better strategies for prevention and treatment of this disease. Adhesion of monocytes to endothelium requires adhesion molecules on monocytes as well as those on endothelial cells [11]. Numerous studies demonstrated that adhesion molecules on endothelial cells are transcriptionally regulated by cytokines, lipopolysaccharide, or other inflammatory mediators [30–33]. In contrast to several adhesion molecules on endothelial cells, overall expression levels of the adhesion molecules on leukocytes appear to be less important in function [11]. Studies so far suggest that activation of leukocytes transforms integrins from an inactive state into an active-avidity state [34–37]. Studies found that avidity of β1 or β2 integrins peaks within 10 min of stimulation and diminishes within 30 min [34].

The β-catenin is a dual-function protein that can function as a transcription regulator in the nucleus or as a component of cell–cell adhesion complexes on plasma membranes [16]. A study by George and colleagues indicated that dismantling of the *N*-cadherin-mediated cell–cell contacts in the vascular smooth muscle cells released β-catenin and induced translocation of β-catenin into nuclei for transcription of cell proliferation genes [26]. This group further demonstrated that translocation of β-catenin coincided with an elevated gene expression of cyclin D1 and

vascular smooth muscle cell proliferation in balloon-injured carotid arteries [35].

In the present study, we analyzed the effects of β-catenin pathway on monocyte adhesion to endothelial cells. Our study demonstrates the following:

1. Activation of the Wnt signaling pathway enhanced monocyte adhesion to endothelial cells (Fig. 1).
2. Elevated levels of β-catenin by GSK-3β inhibitor or proteosome inhibitor MG132 enhanced monocyte adhesion to endothelial cells (Figs. 2 and 3).
3. Enhanced monocyte adhesion to endothelial cells by the β-catenin pathway does not require synthesis of new mRNA or proteins (Fig. 5).

To our knowledge, this is the first report demonstrating that the Wnt/β-catenin pathway enhanced monocyte adhesion to endothelial cells without changing gene expression levels of adhesion molecules. Our study further demonstrated that incubation of monocytes and endothelial cells in the presence of GSK-3β inhibitor for 20 min was enough to enhance monocyte adhesion to endothelial cells (Fig. 4). The results in Fig. 4 are consistent with previous reports that avidity of β1 or β2 integrins in monocytes peaks in 10 min of stimulation [34].

Several signaling pathways have been reported to increase expression levels of adhesion molecules and enhance monocyte-endothelial cell adhesion [8]. However, our study indicated that the β-catenin pathway enhanced monocyte adhesion to endothelial cells without changing expression levels of adhesion molecules. It is likely that the elevated level of β-catenin by the Wnt/β-catenin pathway enhanced monocyte adhesion to endothelial cells as a component of the cell adhesion junction. The enhanced monocyte adhesion to endothelial cells by activation of the Wnt signaling pathway may result from an increase in avidity of adhesion molecules by incorporation of stabilized β-catenin into the plasma membrane.

Our attempt to identify whether activation of the Wnt/β-catenin pathway in monocytes, or endothelial cells, or both is required to enhance monocyte adhesion to endothelial cells did not give clear results. Since avidity of β1 or β2 integrins peaks in 10 min and diminishes in 30 min [34], the essential experimental procedure, treatment of monocytes with GSK-3β inhibitor and rinsing/centrifuging cells twice, markedly decreased monocyte adhesion to endothelial cells.

An increased level of β-catenin by activation of the Wnt signaling pathway has been reported to enhance cadherin-mediated homotypic cell–cell adhesion in a few mammalian cells [38–40]. However, our study demonstrated enhanced cell–cell adhesion between monocytes and endothelial cells by an elevated β-catenin. A previous study found that stimulation of chicken lymphocytes resulted in rapid colocalization of integrin with actin-based cytoskeletons in 20–30 min [41]. The study further indicated that stimulation of mouse T cells resulted in colocalization of LFA with cytoskeleton.

A recent study by Yoshida and colleagues [14] used flow cytometric analysis and demonstrated that treatment of human monocyte U937 for 48 h with cerivastatin, an inhibitor of the cholesterol synthesis pathway, suppressed translocation of RhoA GTPase into the membrane and down-regulated expression of integrin αL, β2, and VLA-4. Since RhoA GTPase pathway has been reported to regulate cytoskeleton organization, it will be interesting to analyze short-term (20–60 min) effects of statins on monocyte adhesion to endothelial cells.

In conclusion, our study demonstrates that the canonical Wnt/β-catenin signaling pathway regulates monocyte adhesion to endothelial cells. Since monocyte adhesion to endothelium is a hallmark of the development of atherosclerosis, identification of a new mechanism for monocyte-endothelial cell adhesion may provide us with strategies to delay or prevent the development of this disease.

Acknowledgments

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